The
# Journal of Infectious Diseases

PUBLISHED BY THE MEMORIAL INSTITUTE FOR INFECTIOUS DISEASES

VOL. 18 — *January, 1916* — No. 1

## THE USE OF BRILLIANT GREEN FOR THE ISOLATION OF TYPHOID AND PARATYPHOID BACILLI FROM FECES *

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While investigating the effect of various aniline dyes on the growth of bacteria[1] we found that brilliant green, aniline green, solid green, smaragd green, and china green, in appropriate dilutions, consistently restrained the growth of the gram-positive bacteria but varied in their action on the gram-negative bacteria. The most important variations were among the members of the colon-typhoid group. The growth of the typhoid bacillus was restrained but slightly, the paratyphoid-enteritidis types grew abundantly, whereas many of the colon group grew feebly or not at all. Many aerogenes types, as well as Bacillus proteus and Bacillus pyocyaneus, were unaffected, even when the dye was present in low dilutions. The concentrations at which this differential action was evident varied with the individual dyes. No one dye seemed more selective than another, but brilliant green exhibited the selective action at higher dilutions.

* Received for publication August 1, 1915.

[1] Krumwiede and Pratt: Jour. Exper. Med., 1914, 19, pp. 20, 501.

On the basis of these results, we suggested the use of brilliant-green broth for the enrichment of typhoid and paratyphoid bacilli in feces. Browning, Gilmour, and Mackie[2] suggested the same procedure for the enrichment of the typhoid bacillus, and Torrey,[3] for the enrichment of the paratyphoid-enteritidis types. The following is a report of the results of the application of this method and of further investigations of the action of brilliant green in fluid and solid media.

It had been found that the addition of feces to brilliant-green broth reduced the activity of the dye. It was necessary, therefore, in order to obtain the point of optimal differential action, either to use graded dilutions of the dye or to vary the amount of feces.

The method (Mr. Lawrence A. Kohn assisted us in carrying out the technical part of this work) employed in examining stools for typhoid bacilli was to add 0.1 c.c. of a moderately heavy suspension of feces to each of 3 tubes containing 10 c.c. of 1% glucose extract broth, neutral to phenolphthalein, to which brilliant green had been added to give final dilutions of 1:300,000, 1:400,000, and 1:500,000. After 15 to 18 hours' incubation, a loop from each tube was streaked on Endo plates, and these examined for typhoid colonies after incubation. Endo plates (Kendall's modification) were also inoculated directly from the suspension of feces used for the dye-broth tubes. The results obtained with this method in routine examinations for typhoid are given in Table 1.

TABLE 1

RESULTS OF EXAMINATION FOR TYPHOID BACILLI IN FECES IN BRILLIANT-GREEN BROTH

| Number of Stools Examined | Result, Endo, Direct Inoculation | Result, Endo, Inoculation from Green-Dye Broth |
|---|---|---|
| 134 | Negative | Negative |
| 26 | Positive | Positive |
| 19 | Positive | Negative |
| 4 | Negative | Positive |
| 183 | | |

In the case of the stools giving positive results with both methods, there was, in some instances, a relative increase of the typhoid bacilli; in others there was a decrease. When stools were positive on direct plating and negative from the dye broth, the loss of the typhoid bacilli was due to the over-growth of dye-resistant organisms, especially the mucoid aerogenes types, and, less frequently, the paratyphoid-like intermediates or members of the proteus and pyocyaneus

[2] Jour. Hyg., 1913, 13, p. 335.

[3] Jour. Infect. Dis., 1913, 13, p. 263.

groups. The following experiment shows how easily such overgrowth may occur.

To 5 c.c. of a boiled suspension of feces were added 1 c.c. of a broth culture of typhoid and an equal volume of a broth culture of Bacillus [lactis] aerogenes. One-half cubic centimeter of this mixture was added to glucose broth containing 1:400,000 of brilliant green. This was plated, incubated, and plated at intervals, to determine the proportion between the two types. Plated immediately, B. aerogenes was to B. typhosus as 5 to 1; after 3 hours, as 9 to 1; and after 24 hours, as 300 to 1.

The work showed that a large proportion of the fecal flora could be suppressed. If this suppression could be obtained by a shorter exposure to the action of the dye, the subsequent loss of the typhoid bacillus by overgrowth of dye-resistant types might be avoided. In this way, the number of typhoid bacilli would be relatively increased altho an actual enrichment due to multiplication would not occur.

Small amounts of suspensions of carrier stools (supplied to us by Dr. Anna M. Agnew of Long Island State Hospital) were added to suspensions of normal stools (to reduce the proportion of typhoid bacilli), and the resultant mixtures used for the following tests. After direct Endo plates had been made, 2 c.c. of the mixture were added to 4 c.c. of broth containing the dye. From this mixture, plates were made immediately and after 1, 3, and 18 hours, respectively. In some instances 2 dilutions of the dye, 1:100,000 and 1:300,000, were employed. No appreciable difference was noted. The results, with the use of a dilution of 1:300,000, are given in Table 2.

TABLE 2

RESULTS OF EXAMINATION FOR TYPHOID BACILLI IN FECES AFTER SHORT EXPOSURE TO BRILLIANT-GREEN DYE

| Number of Specimens | Direct Plates | Plates Made from the Dye Broth at Intervals | | | |
|---|---|---|---|---|---|
| | | Immediately | After 1 Hr. | After 3 Hr. | After 18 Hr. |
| 1 | Negative | Positive | Positive | Negative | Negative |
| 1 | Negative | Negative | Positive | Negative | Negative |
| 1 | Negative | Negative | Positive | Positive | Negative |
| 1 | Positive | Negative | Positive | Negative | Negative |
| 7 | Positive | Positive | Positive | Positive | Positive |
| 3 | Positive | Positive | Positive | Positive | Negative |
| Total 14 | 11 Positive<br>3 Negative | 11 Positive<br>3 Negative | 14 Positive<br>0 Negative | 11 Positive<br>3 Negative | 7 Positive<br>7 Negative |
| Average percentage of typhoid on plates ..... | 50% | 60% | 63% | 45% | 2% |

The shorter exposure to the action of the dye gave a greater increase of positive results over those in direct plating than had been obtained with the previous attempts at enrichment by longer periods

of incubation. The growth on the plates showed, however, that this short exposure is not sufficient to prevent the growth of many of the dye-sensitive types present in the stool when they are subsequently transferred to a favorable medium.

The results with both methods were, on the whole, less satisfactory than had been anticipated. If a dye agar could be devised for direct plating, the suppression of the susceptible types would be more complete than when exposed for a short period to the action of the dye and then transferred to a favorable medium. Various combinations were tried but unsuccessfully, because the typhoid colonies were not sufficiently characteristic to allow of selection when other fecal types developed. It was thought at this time that an indicator was essential to differentiate the lactose-fermenting types. Many were tried but did not give sharp enough differences. The Endo indicator could not be used as the sodium sulfite reduces the activity of the brilliant green. The Andrade indicator, which consists of 100 c.c. of a 0.5% solution of acid fuchsin decolorized by the addition of 16 c.c. of a normal solution of sodium hydrate, was found to answer our purpose. The color of this indicator is restored by acids.

After many trials a medium of the following constitution was found to be satisfactory:

| | |
|---|---|
| Liebig's extract of beef | 3 grams |
| Witte's peptone | 10 grams |
| Salt | 5 grams |
| Agar | 15 grams |
| Water | 1,000 c.c. |

Dissolve in the autoclave. The final reaction must be set to the Andrade indicator. This can be done at the time of preparation, or, as we have found more convenient, the agar can be rendered slightly alkaline to litmus and the final reaction set when used. If the reaction is to be set at once, 100 c.c. are placed in a bottle, 1 c.c. of the indicator added, and the whole titrated to a distinct red color, which will disappear on cooling. Time must elapse between the additions of acid or alkali, as the indicator adjusts itself slowly. The indicator is then added to the remainder of the agar, the reaction adjusted and finished in the ordinary manner. In the method we have employed, because of its greater convenience, the agar is rendered slightly alkaline to litmus, bottled in 100 c.c. amounts, and autoclaved. After determining the acid necessary by testing one bottle, this amount is added to the other bottles as used. This has the advantage that the reaction can be set more accurately in the clear agar and if the first bottle does not give completely satisfactory results, the amount can be varied. After addition of the acid the reaction to phenolphthalein (hot titration) is 0.6% to 0.7% acid.

In either case, the indicator should be added and the reaction adjusted before the sugars and dye are introduced. To the melted agar, just before use, are added 1% of lactose and 0.1% of glucose—sterile 25% solutions of

the sugars being used—and, finally, the appropriate amount of a 0.1% solution of brilliant green. Each 100 c.c. will suffice for 6 plates, as the layer of agar must be relatively thick else the characteristic appearance of the typhoid colony is not as marked. The plates are allowed to stand open until the agar has solidified and then are covered, preferably with porous covers, to absorb the moisture. Inoculation is the same as with Endo plates.

On this medium, many of the fecal types are restrained, whereas the typhoid bacillus produces large colonies which are very characteristic. Viewed through the plate against a dark background, with the light passing obliquely through the agar, they have a peculiar snowflake appearance. Seen by artificial light and a hand lens, under the same conditions, they have the appearance of a coarse wool fabric. They vary slightly in distinctiveness; as a rule, the larger colonies are the more typical and show best where the plates develop a moderate number of colonies. Other fecal types may develop typhoid-like colonies, but this similarity is usually no more troublesome than with Endo or other media.

To test this medium under the most natural conditions obtainable, we added to normal stools small traces of carrier stools. In this way, stools were made containing very few typhoid bacilli. The following results were obtained when suspensions of these mixtures were plated on Endo and on the green-dye agar:

| | |
|---|---|
| Total examined | 130 |
| Endo agar | 71 negative; 59, or 45%, positive. |
| Green-dye agar (0.2 c.c. of a 0.1% solution of dye to 100 c.c. of agar) | 24 negative; 106, or 81%, positive. |
| Increased positive results | 36%. |

Of the 71 negative on Endo, 47 (or 67%) were positive on the green-dye agar. None was positive on Endo when negative on the dye agar.

Fifty-seven samples were also added to green-dye broth as in the preceding experiments, and, after one-half hour, plated on Endo. Of these, 8 were positive, altho negative on the direct Endo plates, but 6 were negative which on the direct Endo plates had been positive. Twenty-two were negative which had been positive on the direct green-dye plates. None was positive which on the direct green-dye plates had been negative.

Sixty-six samples were enriched in the same way, but plated on green-dye agar instead of Endo. Three were negative which had been positive on the direct green-dye plates and 3 were positive which

on the direct green-dye plates had been negative—as a result, in two instances, of the fact that the direct plates were overseeded.

At the same time 0.1 c.c. of the suspension was inoculated into 10 c.c. of glucose broth containing 1: 300,000 of the dye, and after 18 hours' incubation plated on both Endo and green-dye agar. Forty-seven samples were tested. Six in both cases gave positive results which on the direct Endo plates had been negative, but 30 were negative which on the direct green-dye plates had been positive. In only one instance was the sample positive which on the direct green-dye plates, as well as on the Endo plates, had been negative.

While the comparative examinations described were being made, it was noted that with certain stools the number of colonies developing on the green-dye agar was only slightly less than on the Endo agar. Considerable difficulty was encountered in some instances because of the similarity of some of the colonies to those of the typhoid bacillus. These colonies were fished and found to be either intermediate types, slow lactose fermenters, or incompletely restrained colon types. It appeared to us that we might exclude these organisms with stronger dilutions of the dye. Altho the number of typhoid colonies was decreased, they should be more easily found, were the typhoid-like type not present. Comparative tests showed that the concentration of the dye could not be carried much farther than 1: 300,000, else the typhoid bacillus might not develop. From these tests it was decided to prepare the agar as before, but to use 2 series: one to contain 0.2 c.c., the other 0.3 c.c. of a 0.1% solution of the dye to 100 c.c. of the agar. In this series of comparative tests, Conradi's brilliant-green agar, of which we shall speak later, was also included. The material used for inoculation was a mixture of carrier and normal stools. The results are given in the following table:

TABLE 3

RESULTS OF EXAMINATION FOR TYPHOID BACILLI IN FECES EXPOSED TO DIFFERENT CONCENTRATIONS OF BRILLIANT GREEN

| Result | Endo | Amount of 0.1% Dye Solution for 100 c.c. of Agar | | Conradi |
|---|---|---|---|---|
| | | 0.2 c.c. | 0.3 c.c. | |
| Negative | 18 | 9 | 3 | 14 |
| Positive | 10 | 19 | 25 | 14 |
| Average total of colonies developing | 1,250 | 230 | 70 | 40 |
| Average number of typhoid colonies | 20 | 40 | 25 | 10 |

In one instance the agar containing 0.2 c.c. of the dye was positive, whereas the agar containing 0.3 c.c. was negative, because of almost complete inhibition of growth. In no instance were the Endo or the Conradi plates positive when our green-dye agar was negative. The figures given of the number of colonies developing on the different media are only roughly comparative, as more material was inoculated on the media containing the dye. They serve, however, to give an idea of the amount of restraint of the ordinary fecal types and the relative enrichment of the typhoid bacilli.

About this time a small, localized epidemic of typhoid occurring, we received 9 stools for diagnosis. Of these, none gave positive results on Endo medium, 4 were positive on the green-dye agar containing 0.2 c.c. of dye, and 5 were positive on the agar containing 0.3 c.c. of the dye. These natural stools acted as a control on the results obtained with normal stools inoculated with carrier stools.

The tests in this series were severe, as the stools were especially rich in dye-resistant types. When typhoid-like colonies developed on the agar containing less dye, they were in great part excluded on the agar containing more dye.

Table 4 gives the results of the routine examination of feces, chiefly from convalescents prior to discharge. This includes the specimens mentioned.

TABLE 4

RESULTS OF ROUTINE EXAMINATION FOR TYPHOID BACILLI IN FECES

| Number Examined | Endo | Green-Dye Agar | |
|---|---|---|---|
| | | 0.2 c.c. | 0.3 c.c. |
| 631 | 65 positive | 89 positive | 89 positive |
| | | 3 positive | 3 negative |
| | | 7 negative | 7 positive |
| | | Total positive 99 | |

In two instances the typhoid bacilli in the stools were unusually sensitive to the dye, as shown by the development of small to fine colonies. In spite of this, one of the samples was positive on the dye plates only.

In the use of the green-dye agar as described, many points have arisen as to the importance of the different constituents, which have been made clear by a number of comparative tests. We can best show these points by discussing each.

The concentration of the agar cannot be varied from that given without finding a new optimal concentration of the dye. Thus, if the dye be used with 3% agar, there is a marked decrease in its action.

The concentration of the peptone and meat extract has been found satisfactory, and no variations have been tried. By analogy with other observations, we are certain that any increase in the concentration will be accompanied by a decrease in the activity of the dye.

The trace of glucose was added because we had previously found that this amount of sugar would markedly increase the size of cholera colonies. As this amount of glucose is employed in the Russell medium, we did not anticipate that there would be sufficient acid produced by surface colonies to affect the indicator. The presence of this trace of glucose is the fundamental factor in the success of our medium. The typhoid colony is usually larger and more important; its distinctiveness is increased. Large colonies are desirable, as they furnish ample material for macroscopic slide agglutinations, which allow a rapid survey of many colonies. When the reaction of the medium is set close to the neutral point of the indicator, the use of glucose is followed by a slight tinging of the colony, which, however, greatly increases its characteristicity. Comparative tests without glucose have given very poor results because of the lessened distinctiveness of the typhoid colony.

The indicator has been tried in different concentrations in the hope of accentuating the lactose-fermenting types. On the whole, this has made no appreciable difference and we have decided to employ 1%.

The lactose was added to differentiate the lactose-fermenting types. As many of them are inhibited, this is not so important as we at first considered it. Furthermore, most of the lactose-fermenting organisms which develop are either distinctive in their colony morphology (as the aerogenes types), or produce insufficient acid to affect the indicator. Similar results are noted in the use of the Endo agar, in which many colorless or nearly colorless colonies may develop, which, on isolation, are found to be lactose fermenters. On this account it might seem that the lactose and the indicator could be omitted, reliance being placed wholly on the distinctiveness of the typhoid colony. Such attempts, however, in many comparative tests, have shown very poor results. Many of the lactose-fermenting types simulate the colony of the typhoid bacillus so closely that great difficulty is encountered; this is not the case with plates inoculated with the same material, but with the indicator and the lactose present. As has been noted, if colon types develop partially on the dye agar, they will simulate the typhoid colonies. Similarly, many types which develop with a high dilution of the dye, but fail to develop at lower dilutions, show a typhoid-like colony. It would seem then, that the lactose aids in promoting the maximal development of those lactose-fermenting types which are able to develop at all. When this is the case, they do not resemble the typhoid colonies, altho they may when their growth is restrained. This deduction seems warranted, as the greatest trouble is noted with the weaker dye agar, when lactose is omitted, whereas the troublesome types are excluded to a great extent on the stronger dye agar. As has been noted, the slight color developing in the typhoid colonies adds to their distinctiveness, but should it be absent, typhoid-like colonies are, as a rule, not confusing unless the lactose be omitted.

Some of the slow lactose-fermenting types which, under certain circumstances, develop typhoid-like colonies, change the indicator very slightly. This change, however, is sufficient to rob them of most of their typhoid-like appearance.

The reason for the 2 concentrations of the dye has already been fully discussed. The results show that both dilutions are necessary, as the stronger may give so great a degree of inhibition that the typhoid bacilli are lost.

One brand of brilliant green has been used in most of this work. Considerable variation is noted at times with certain solutions of the dye or certain batches of agar.. A cloudy agar with sediment cannot be used, as the dye loses a large part of its activity. Different samples of the same brand of dye probably vary and the one brand of dye may not always be available. For this reason it was necessary to determine some method of standardization that could be used not only to test a sample of dye, but also to check the concentration of a dye solution with each batch of agar. When many routine examinations are being made, this takes comparatively little time, and the necessity of repeating the tests can be avoided by preparing the agar in large amounts.

Various methods for standardization were employed, using carrier stools, various freshly isolated strains, as well as stock cultures of typhoid bacilli. As the amount of extraneous matter in the stools was a disturbing factor and the various strains showed some variations in resistance, the Rawlings strain, which is available in most laboratories, was finally adopted for the comparative tests. The amount of culture inoculated is important, as overseeding will obscure the gradations in the activity of the dye. Table 5 shows the results of a few standardization tests with varying amounts inoculated, these results serving as examples of the action of a correctly balanced dye agar.

TABLE 5
RESULTS OF STANDARDIZATION TESTS

| Endo | Amount of Dye to 100 c.c. Agar | | |
|---|---|---|---|
| | 0.2 c.c. Dye | 0.3 c.c. Dye | 0.5 c.c. Dye |
| Thousands | Thousands | Thousands | 75% reduction |
| Thousands | Thousands | Slight reduction | 90% reduction |
| 2,000 | 2,000 | 1,500 | No growth |
| 200 | 200 | 180 | No growth |
| 60 | 60 | 50 | No growth |
| 40 | 40 | 35 | No growth |
| 30 | 25 | 6 | No growth |

In making the tests, the plates are streaked, but with a little practice fairly uniform inoculations can be obtained. In obtaining dilutions for inoculation, we have found that 0.5 c.c. of a broth culture diluted with 10 c.c. of broth will contain 2,000 to 5,000 bacilli per small loop. If a small loop of a broth culture be added to 10-15 c.c. of broth, a loop will contain from 25 to 200 bacilli.

If the dilutions given are tried, it is a simple matter to test out a sample of dye or a batch of agar. Should variations be noted from the results given, proportionate changes in the final dilutions of the dye to be employed can easily be determined. The fact that 2 dilutions are used compensates for slight variations.

For an occasional examination with an untested sample of dye the use of 0.2, 0.3, and 0.4 c.c. of the dye to 100 c.c. of agar would probably cover the range of action of most samples of brilliant green or variations in media.

Three samples of dye, from Bayer, Grübler, and Höchst, have been compared by this method, 2 solutions being used of each dye made from different

parts of the sample. The variations between the different dyes were moderate and no greater than between the two solutions made from the same dye. With careful standardization it would seem, therefore, that any of these preparations could be used. If the test against the Rawlings strain gives correct results as far as we can see, the restraining action upon the fecal flora will be satisfactory. The degree of restraint of the fecal flora varies with different stools. The degree of restraint is, as a rule, greater with fresh specimens. In becoming familiar with the medium, we strongly advise the use of stools artificially inoculated with traces of carrier stools or with typhoid culture. The use of pure cultures may give misleading results, as fecal strains belonging to the B. coli group vary widely.

The dye solutions keep fairly well, but there seems to be some irregularity in their rate of deterioration. We have used some solutions as long as 2 months. It would be well, however, to re-test a solution every 2 weeks as a safeguard against deterioration. The poured plates keep for several days. We have not tested this point as it is relatively unimportant to those doing routine examinations.

## ROUTINE METHOD NOW EMPLOYED

A large sample of the stool to be examined is rubbed up in extract broth and diluted with broth to a density (roughly a 1:15 dilution by volume of a formed stool) which, experience has shown, will give discrete colonies when a loopful is streaked over 2 successive Endo plates. The suspension is allowed to stand so that particles of feces will settle. One loop of the suspension is placed on a 0.2-c.c. and on a 0.3-c.c. plate; these are streaked in the order given and then an Endo plate. Two loops are placed on each of a similar pair of green-dye plates and streaked in the same order, and last an Endo plate. The wire used for streaking is not flamed while inoculating the series of plates. For practical routine examinations this seems a sufficient number of plates to employ. Altho the Endo plates have never been positive when the dye agar was negative, we do not feel with our present experience that they can be omitted. Unusually sensitive strains are a possibility.

Some of the details of our technic might be described, as we find that they are not generally known. We do not employ glass rods to inoculate the plates, as they are unwieldy and cool too slowly after sterilization, but a suitably bent, heavy platinum wire with a loop at the end. A loop of the material is carried across the center of the plate and then smeared over the plate with the flat part of the wire, the plate being turned so that the entire surface is sown. A second plate can be inoculated in the same fashion without burning the wire. If 3 such wires are employed and used in rotation, they cool before being required again.

For a direct tentative agglutination from the plate, the macroscopic slide method is employed. A small drop of salt solution and one of an agglutinating serum are placed on a slide. The suspicious colony is taken up with a very small loop and rubbed first in the salt solution and then in the serum. A positive result is shown by immediate clumping, whereas the salt control remains evenly clouded. If spontaneous agglutination occurs, this will be noted in the control. A high titer serum is essential and the dilution must be lower than that ordinarily employed, as the reaction must take place very rapidly or the drop will dry. The optimal dilution must, of course, be determined for each sample of serum. As the fecal strains of typhoid are more agglutinable than blood strains, inagglutinable strains are not so apt to be encountered. One peculiar source of error has been noted, however. Certain strains have shown a prezone phenomenon; that is, they agglutinated poorly or not at all at low dilutions, but agglutinated well at higher dilutions. However, this method, with a known serum, gives results that are very reliable and we give a tentative diagnosis on this basis. Unknown sera, especially if they be fresh, may lead to serious error unless thoroughly tested with various types developing on the plates, as well as with known strains of allied bacilli.

For fishing, the Russell medium is employed with the following modification. In place of litmus, 1% of the Andrade indicator is used, and the reaction set accordingly. This modified medium gives much sharper results, and the acid production in the butt is very evident; the indicator does not decolorize, as is the case with litmus. As the medium is rather delicate, each new lot should be tested, before use, with a few known strains, to be sure that it is not too sensitive.

In fishing from the green-dye agar there is the possibility of not obtaining pure cultures. This is a disadvantage of all differential restraining media, the restrained bacteria being able to grow when transferred to a favorable medium. It is well to save suspicious plates until the fishings are inspected, or to replate from them immediately.

Besides inoculating plates in the manner described, 0.1 c.c. of the fecal suspension may be added to 1% glucose extract broth, neutral to phenolphthalein, containing 1:300,000 of the dye. If, as occasionally happens, very little growth develops on the direct dye agar and no typhoid is found, Endo agar is inoculated from the broth tube after 18 hours' incubation. This is most likely to be successful when the flora is dye-susceptible, giving an added chance of success. Should

the dye agar, however, show abundant growth, sub-inoculations from the dye-broth tube seem superfluous.

There would seem to be no reason why these results cannot be applied to the isolation of members of the paratyphoid-enteritidis groups. We have had only one naturally infected specimen,—a sample of soup which produced gastro-enteritis in several members of a family. When this was plated on Endo, about 1,000 colonies developed, of which 1 was a paratyphoid type. On green-dye plates of the usual strength, only a few paratyphoid types developed.

Pure cultures of this group were plated with seeding well under 150 for a plate, to determine whether appreciable restraint would be evident on the green-dye agar of 0.2, 0.3, and 0.5 c.c. strength.

Most of the strains showed no, or at most slight, inhibition on the strongest dye agar. Some showed a moderately marked inhibition, and a few failed to grow on the 0.3 c.c. dye agar. A few showed inhibition even on the 0.2 c.c. dye agar. This agrees with our previous conclusion[2] that the members of this group have a variable sensitiveness to brilliant green, altho most of the strains are extremely dye-resistant.

The colony morphology is variable. The more feebly growing types are typhoid-like; the more vigorous strains produce 2 kinds of colonies with intermediate gradations. Both types are larger than the typhoid colony. One type of colony is yellowish-white, raised, and moist. The denser center is homogeneous, but the edge shows some markings similar to those in the typhoid colony. The other type is flattened, spreading, feathery, and irregular in outline, the typhoid-like markings being exaggerated. Some strains produced both types of colonies, and fishings on replating bred true.

The use of dye broth for enrichment is more likely to be successful with the resistant members of this group, as stronger dilutions of the dye may be employed.[2]

We have not discussed the results of previous investigations of the application of aniline dyes for the enrichment of the typhoid-paratyphoid group. Our own work developed independently on the basis of our observations of the action of the various aniline dyes. As the outcome has shown, it is fortunate that we did not attempt merely to modify existing media. We have tested several of the methods suggested, with results inferior to our own. No attempt was made to try out the various malachite-green media, as our results have shown that this dye is inferior to brilliant green in differential action. Furthermore, different samples of malachite green have been found to vary widely in their action, whereas most samples of brilliant green give similar results.

As the Conradi brilliant-green agar[4] approached most nearly the medium we have devised, we have employed it in comparative tests. The results, given in the preceding tables, are not very favorable to Conradi medium. Furthermore, this latter, containing 3% of agar (which seems to us unnecessary), and 20 grams of meat extract to the liter (whereas we used only 3), is more difficult to make and more expensive—both factors of importance in routine work. On the whole the Conradi medium is too restraining; the colonies are somewhat characteristic but insufficiently so. German workers agree with us in these points. The Conradi agar is now chiefly employed as a preliminary enrichment medium. The plates are heavily inoculated and after incubation are flooded with broth, whereupon the typhoid colonies, less adherent than other types, float off easily, and the suspension is inoculated either on Conradi-Drigalsky medium or on Endo. We have tried this method with different green-dye media, and altho the results were better than from the use of Endo medium and the usual technic, they were not as good as with our medium alone. Furthermore, this method delays the results one day.

China green has been employed by Werbitski, and some workers have found it superior to the Conradi brilliant-green medium as a preliminary enrichment medium. We did not find China green more differential in its action than brilliant green; moreover, its action is not evident at equally high dilutions. For this reason, we have not tried this medium and are inclined to attribute any better results obtained in its use as referable to a better adjustment of the dilution of the dye to the other constituents rather than to any inherent superiority in the dye itself.

The attempts of various workers[4] to use a differentially selective dye decolorized by sodium sulfite and designed to act both as a restraining agent and indicator, are, in our opinion, due to a misconception of the mode of action of the dyes employed. We have found it impossible to balance the degree of loss of action of the dye due to sodium sulfite. We have also employed caffein, as advised by some investigators, with only moderate success.

It would require too much space to analyze even a small part of the literature which exists on typhoid isolation. The mere existence of a great number of methods is evidence of the unsatisfactory results obtained. On the whole, however, the application of the selective media described in this paper has been very successful in the isolation of the more resistant paratyphoid-enteritidis group.

## SUMMARY

With an easily prepared agar, 2 concentrations of brilliant green being used, it has been possible markedly to increase the percent of positive typhoid isolations from stools. This has given much better results than the employment of brilliant-green broth alone, altho the dye-broth enrichment method may give positive results when the agar alone fails. The methods can be applied to the isolation of the members of the paratyphoid enteritidis group from stools.

[4] Kutscher: Kolle and Wassermann, Handb. d. pathogen. Mikroorganismen, 1913, 3, p. 717.